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Efficacy of raw milk preservation by aqueous extracts of Tulsi (*Ocimum sanctum*) and Neem (*Azadirachta indica*)

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ABSTRACT

The study was aimed at assessing the effect of herbal plant leaves aqueous extract in prolonging the shelf-life of raw cow milk. Tulsi (*Ocimum sanctum*) and Neem (*Azadirachta indica*) were selected based on phytochemical analysis for the presence of phenolic compounds. The presence of phenolic compounds in the leaves extracts were confirmed qualitatively. Initial physicochemical and sensory attributes of raw cow milk were also performed. Both 1.25 and 1.5% Tulsi extract treated samples were found to be of normal yellowish-white color up to 19 h of storage. On the contrary, the 1.5% Neem extract was found to show the same color at 19 h of preservation. A significant difference ($p<0.05$) was found in the acidity, pH and clot-on-boiling (COB) test among the samples. The most acceptable acidity was found up to 14 h at the concentration of 0.75, 1.25 and 1.5% of Tulsi extract. In the case of Neem extract, the satisfactory acidity was noted for 1.25% concentration up to 17 h of storage. The most agreeable pH (6.5 to 6.8) of raw milk was recorded for 1.5% Tulsi and 1.5% Neem extract up to 17 h and 18 h, respectively. A concentration of 1.25% and 1.5% Neem extract was recorded as COB negative up to 15 h and 16 h of preservation, respectively. In response to microbial analysis, the lowest number of the total viable bacterial count was recorded for 1.25% Tulsi and 1.5% Neem extract with 3.79±0.87 and 3.62±0.90 $\log_{10}$CFU/mL. In conclusion, comparing the Tulsi and Neem extract in response to raw milk preservation both Tulsi and Neem extracts can be used with 1.5% concentration for up to 17 h of storage at room temperature.

Introduction

Since time immemorial, milk has been a part of the human diet. It meets the nutritional requirements of the human body perfectly than any other single food. There is no alternative to milk yet exists as an ideal food (Mela, 1999). However, milk is highly perishable because it is an excellent medium for the growth of microorganisms (Singh *et al*., 2011). Thus, special measures and concerns are essential to confirm that it reaches the market in a satisfactory condition. Consequently, the preservation of milk in subtropical countries like Bangladesh is a major challenge, especially where refrigeration facilities are limited and high environmental temperature (>30^{o}C).

Lactoperoxidase (LP) is a glycoprotein that has been proven to be effective against both gram-positive and gram-negative microorganisms presents by nature in colostrum and milk (Kussendrager and van Hooijdonk, 2000; Seifu *et al*., 2005; Boots and Floris, 2006). LP system becomes active with the presence of hydrogen peroxide and thiocyanate. Therefore, a way has been followed to preserve the milk with chemical preservatives (hydrogen peroxide, formaldehyde, sodium carbonate/bicarbonate, mercuric chloride, potassium dichromate, etc.) either act as direct microbial poisons or

reduce the pH to a level of acidity that prevents the growth of microorganisms (Singh *et al*., 2015). Nonetheless, the use of chemical preservatives is quite debatable due to their harmful residual effects on human health (Cerdán *et al*., 2010).

At present, a growing interest to use natural antimicrobial compounds like extracts of herbs and spices for the preservation of food has emerged. The mode of action of natural preservatives is inhibition of microbial growth, oxidation and certain enzymatic reactions arising in milk (Kroger, 1985). The use of plant extracts as a source of phenols is preferred as a natural method (Gad and Salam, 2010). Phenols and polyphenols are water-soluble compounds that can be easily mixed with milk. Several investigations analyzed the phytochemical parameters and recorded that water extracts of Tulsi (*Ocimum sanctum*), Neem (*Azadirachta indica*) and other related species contain glycosides, tannins, and alkaloids (Jeyaseelan *et al*., 2010; Joshi *et al*., 2011; Shafqatullah *et al*., 2013; Del Serrone *et al*., 2015). In addition, herbs like many other plants readily synthesize phytochemicals that protect against attack by

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insects, herbivores, and microorganisms (Rao *et al*., 2010). Rokhsana *et al*. (2007) observed that raw cow milk immersed with water hyacinth leaves in an open space with stirring extended the shelf life of milk for 5 h. Besides, Abdulla *et al*. (2007) reported that addition of mango seed kernel extract into milk decreased the pH from 6.6 to 6.1 when compared to control (6.6 to 4.7) in 8 h. Addition of 0.5 percent Tulsi leaf extract (v/v) to raw milk remained acceptable up to 11 h of storage at 37°C (Sivakumar, 2017). On the contrary, the antimicrobial activity of Neem leaf against spoilage bacteria is very promising and potentially inhibits *Penicillium verrucosum* and *P. brevicompactum* growth, sporulation and mycotoxin (Mossini *et al*., 2009). The herbal plant extracts contain a number of phytochemicals which are well-known to retard microbial growth and oxidative degradation thus improving the quality and nutritional value of foods. The presence of these phytochemicals has made herbs be of great interest to the food industry. These natural preservatives are gaining importance in recent years as they have no harmful effects on human health (Tuormaa *et al*., 1994; Carocho *et al*., 2018; Mangang *et al*., 2020).

However, to the best of our knowledge, a few works have been reported on the preservation of milk by using herbal plant extracts. Thus, the present study was undertaken to investigate the efficacy of Tulsi and Neem leaf extracts on the shelf life of raw milk at room temperature

Materials and Methods

The experiment was conducted at the Laboratory of Dairy Microbiology and Biotechnology, Department of Dairy Science, Bangladesh Agricultural University (BAU), Mymensingh-2202, Bangladesh.

Milk sample

Fresh raw milk was collected from BAU Dairy Farm. All hygienic measures were strictly maintained by advising the milkmen. The initial physicochemical quality of raw milk was measured by Milk Analyzer (Lactoscan, Nova Zagora, Bulgaria) and organoleptic test (appearance, color, texture, consistency and flavor) and the data is presented in Table 1. Sensory attributes were evaluated using a 9-point hedonic scale and values ranged from 1-9, wherein: (1) extremely unpleasant, (2) very unpleasant, (3) moderately unpleasant, (4) slightly unpleasant, (5) neither pleasant nor unpleasant, (6) slightly pleasant, (7) moderately pleasant, (8) very pleasant and (9) extremely pleasant (Seczyk *et al*., 2016).

Preparation of Tulsi and Neem leaf extracts

Tulsi (*Ocimum sanctum*) and Neem (*Azadirachta indica*) leaves were collected from Botanical Garden, BAU, Mymensingh. The fresh mature Tulsi and Neem leaves were selected for extract preparation. The leaves were washed and shade dried. The dried leaves were grounded individually through 1mm sieve (Thomas Wiley Mill 4, Minnesota, USA) and the powder was kept in airtight containers. According to the method of Preethi *et al*. (2010), 10 g of Tulsi and Neem powder was separately mixed with 100 mL of distilled water in the beaker. The mixtures were kept for 24 h for soaking and next day the mixtures were filtered through Whatman No. 4 filter paper. Finally, the extract was diluted with sterile distilled water to a concentration of 100 mg/mL and then kept in refrigerator (4^{o}C).

Table 1. Initial physicochemical and sensory attributes of raw cow milk

Parameters	Observations
Fat (%)	4.50±0.25
Protein (%)	3.60±0.08
Lactose (%)	4.10±0.23
TS (%)	12.55±0.70
Specific gravity	1.034±0.01
Acidity (%)	0.116±0.09
Clot on Boiling Test (COB)	Negative
[1]Sensorial attributes	
Appearance	Uniform (8.5)
Color	Yellowish white (8)
Texture	Floating fluid (8)
Consistency	Uniform (8)
Flavor	Pleasant aromatic (8)

Qualitative determination of the presence of phenolic compounds

According to the method suggested by Himaja *et al*. (2010), the presence of total phenolic compounds in Tulsi and Neem leaf was determined qualitatively (Table 2). In brief, 2 mL of extract was diluted with an equal volume of distilled water. Afterward, a few drops of 5% ferric chloride solution were added to 2 mL of diluted extract. A violet color was considered as the presence of total phenolic compounds. Again, 2 mL of extract was reacted with a few drops of 98% hydrochloric acid and filtered. A few drops of Mayer's reagent were added to 1 mL of filtrate, subsequently, a cream color precipitation indicated the presence of alkaloids.

Table 2. Qualitative test for the presence of phenolic compounds

Presence of	**Tulsi (*Ocimum sanctum*)**	**Neem (*Azadirachta indica*)**
Phenolic Compounds	Positive	Positive
Alkaloids	Positive	Positive

Experimental layout

A volume of 100 mL milk sample was taken in 9 different test tubes. The tubes were labeled according to the test groups. The tubes were arranged for incorporation with aqueous extract of Tulsi (0.25%, 0.75%, 1.25% and 1.5%) and Neem (0.25%, 0.75%, 1.25% and 1.5%) along with a Control (no extract) group. The samples were observed for preservation at room temperature (30±1.5^{o}C) up to spoilage.

Physicochemical analysis

Titratable acidity was measured against 0.1 N NaOH and phenolphthalein indicator (AOAC, 2005). The pH value was measured using a digital portable pH meter (Hanna, Romania).

Shelf life assessment of raw milk in response to Tulsi and Neem extract

As far as shelf life determination is concerned, clot on boiling test (COB) as per BIS (1981); titratable acidity (AOAC, 2005) and pH (Hanna, Nova Zagora, Romania) were performed. The microbial analysis (total viable counts, TVC) was determined by the standard plate count method according to APHA (2004). In short, Plate count agar (PCA agar, Himedia, India) was used for viable bacteria count. Plate count agar media and sterile saline (0.9% NaCl, w/v) were prepared. The samples (1 mL) were transferred into 9 mL sterile saline and serially diluted up to 10^5, then 1 mL diluted sample put into plate and poured agar (10-15mL) and allowed for solidification. After that, the plates were incubated at 32 °C for 48 h. The colonies were enumerated for the plates which had 30-300 grown colonies.

Statistical analysis

The data obtained regarding titratable acidity, pH, total viable count were statistically analyzed for one-way ANOVA. Tukey test were used for mean comparison. The analysis was performed by using SPSS Windows package program Version 20.

Result and Discussion

Shelf life

The experimental results regarding milk color, flavor and clot-on-boiling (COB) test are stated in Table 3 and 4, respectively. It is evident from the data that the color of milk was yellowish white up to 8 h irrespective of the treatment group. In case of raw milk treated with both 1.25 and 1.5% Tulsi aqueous extract was found normal yellowish-white color up to 17 h. In contrary, the 1.5% Neem extract was found to show the same color up to 19 h of preservation. The others treated group was appeared as bleached color at the same condition. Thus, it can be stated that the Neem extract was more satisfactory in respect to milk color during preservation at room temperature (30 ± 1.5°C).

Table 3. Effect of aquous extract of Tulsi and Neem on color of raw milk during preservation

Hours	Control	Tulsi (%)				Neem (%)			
	(0%)	0.25	0.75	1.25	1.5	0.25	0.75	1.25	1.5
0	yw[1]	yw	yw	yw	yw	yw	yw	yw	yw
3	yw	yw	yw	yw	yw	yw	yw	yw	yw
6	yw	yw	yw	yw	yw	yw	yw	yw	yw
8	yw	yw	yw	yw	yw	yw	yw	yw	yw
9	b	yw	yw	yw	yw	b	yw	yw	yw
10	b	yw	yw	yw	yw	b	b	yw	yw
11	b	b	yw	yw	yw	b	b	yw	yw
12	b	b	yw	yw	yw	b	b	yw	yw
13	b	b	yw	yw	yw	b	b	yw	yw
14	b	b	yw	yw	yw	b	b	yw	yw
15	b	b	yw	yw	yw	b	b	b	yw
16	b	b	yw	yw	yw	b	b	b	yw
17	nd	nd	b	yw	yw	b	b	b	yw
18	nd	nd	nd	b	b	b	b	b	yw
19	nd	nd	nd	nd	nd	nd	nd	nd	yw
20	nd	nd	nd	nd	nd	nd	nd	nd	b

[1]yw = yellowish white; b =bleached

Table 4. Effect of Tulsi and Neem aquous extract on flavor of raw milk during preservation

Hours	Control	Tulsi (%)				Neem (%)			
	(0%)	0.25	0.75	1.25	1.5	0.25	0.75	1.25	1.5
0	p	p	p	p	p	p	p	p	p
3	p	p	p	p	p	p	p	p	p
6	p	p	p	p	p	p	p	p	p
8	p	p	p	p	p	p	p	p	p
9	ss	p	p	p	p	p	p	p	p
10	s	p	p	p	p	s	p	p	p
11	s	p	p	p	p	s	p	p	p
12	s	ss	p	p	p	s	ss	p	p
13	s	s	p	p	p	s	s	p	p
14	bit	s	s	p	p	bit	s	s	p
15	bit	s	s	p	p	bit	s	s	p
16	bit	bit	s	s	p	s	s	ss	p
17	nd	nd	nd	nd	nd	s	s	ss	p
18	nd	nd	nd	nd	nd	nd	nd	s	ss
19	nd	nd	nd	nd	nd	nd	nd	nd	ss
20	nd	nd	nd	nd	nd	nd	s	s	s

p = pleasing; ss = slight sour; s = sour; bit = bitter and nd = not determined.

In response to flavor of milk treated with 1.5% Tulsi extract was found pleasing flavor up to 16 h but the control group was became sour at 10 h of preservation. In the other hand, Neem extract with 1.5 % concentration was noted for pleasing flavor up to 17 h without showing any Neem-like odour. Comparing the Tulsi and Neem in response to raw milk preservation the Neem with 1.5% concentration was found best in terms of color and flavor. Nevertheless, the raw milk can be preserved with acceptable color and flavor from 16 to 17 h at room temperature by using both Tulsi and Neem extracts. Similar results were reported by Sivakumar, (2017) in case of Tulsi and Abdulla *et al*. (2007) when treated milk samples with mango seed kernel. The changes of color and flavor during preservation can be attributed to the microbial population and developed acidity of raw milk. Raw milk microflora is multiplied favorably at room temperature condition. Consequently, the raw milk acidity is increased over time proceed by developing bleach color and sourly flavor (Kang *et al*., 2010; Campbell *et al*., 2012; Sivakumar, 2017). The acid production of the milk bacteria can be delayed by the phytochemicals present in Tulsi and Neem leaf aqueous extracts consequently increased shelf life up to 8 to 9 h (Joshi *et al*., 2011).

Acidity, pH and COB test

A significant difference ($p<0.05$) was found in the acidity of all samples (Fig. 1). The most acceptable acidity (0.17%) was found up to 14 h at the concentration of 0.75, 1.25 and 1.5% of Tulsi extract. In case of Neem extract, the acceptable acidity (0.16%) was noted for 1.25% concentration up to 17 h of preservation. The highly significant difference within treatment may be due to presence of the bacteria which were capable of increasing the acidity during the log phase (Urbiene and Leskauskaite, 2006). The highly significant difference between treatments may be due to the presence of phenolic compounds which were able to overcome the inhibitory effect of compounds in the food matrix as mentioned by Ersoz *et al*. (2011). From the Fig. 1, it was observed that untreated raw milk was acceptable only up to 8 h with titratable acidity of 0.17% after that the acidity was gradually increased. These results are in agreement with the findings of Abbas and Osman, (1998) who reported that the titratable acidity in milk increased gradually during preservation.

The pH of the raw milk kept at 30°C showed a decreasing trend in the control group. Significantly acidic milk was noted at 13 h of preservation. The most satisfactory pH (6.8) of raw milk was recorded for 1.5% Tulsi and 1.5% Neem extract up to 17 h and 18 h, respectively (Fig. 2). The observations were in accordance with Abdulla *et al*. (2007) who reported that the addition of mango seed kernel extract into milk decreased the pH of the treated sample slower when compared to control. The declining trend of pH might be due to the inhibitory effect of phytochemicals of the extract used on the microbes.

The longer shelf-life of raw milk was observed up to 14 h at 1.5% Tulsi aqueous extract. These findings were found similar to that of Ray (2008) who found that 1.0% banana pseudo stem juice increased the shelf life up to 12 h of milk preservation at 30 ± 2°C. Moreover, COB test was found negative up to 11 h in all four groups of Tulsi treated samples (Table 5). In comparison with Neem extract the result showed that 1.25% and 1.5% concentration was recorded as negative up to 15 h and 16 h of preservation, respectively. It is worth to note that the raw milk can be preserved 6 to 7 h more compare to control group which is almost similar to the observation of Chakraborty *et al*., (2011) and Jandal (1996).

Microbial analysis

Microbiological analysis of raw milk was conducted to determine the effect of the aqueous extract of Tulsi and Neem on the microbial quality of milk during preservation at room temperature. Data regarding total viable bacterial counts (TVC) are shown in Table 6. The results revealed that the bacterial population in the control group was gradually increased by an undeviating fashion in advancement of the preservation period. The TVC of the untreated samples showed a gradual increase, whereas the treated samples showed a retarded increase of TVC up to 20 h of storage. This correlates with the findings of Ray (2008) who found the restricted increase in TVC during preservation of raw milk treated with banana pseudostem juice. The lowest number of bacteria was recorded for 1.25% Tulsi and 1.5% Neem extract with 3.79±0.87 and 3.62±0.90 $\log_{10}$CFU/mL. It is evident from the Table 6 that addition of Tulsi and Neem extract could inhibit the multiplication of bacterial population in the raw milk samples. The restricted increase in standard plate count in treated samples may be due to the phenolic compounds possessing broad spectrum of antimicrobial activity present in the extracts (Chandra *et al*., 2012; Rahmanisan *et al*., 2015). The action involves interaction of the cytoplasmic membrane with phenolic compounds and activity is selectively higher against gram positive bacteria (Sharma *et al*., 2010). However, the restriction in the bacterial growth might be attributed to antimicrobial activity of phenolic compounds which causes the destruction of bacterial cell wall and cytoplasmic membrane resulting in the leakage of cytoplasm (Shan *et al*., 2007).

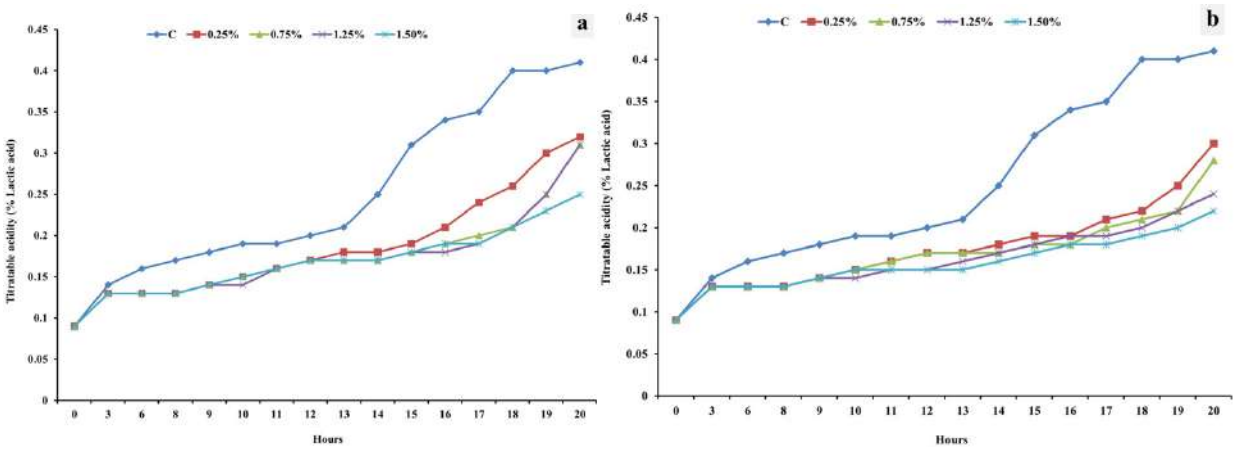

Fig. 1. Changing pattern of titratable acidity during preservation of raw milk treated with leaf aqueous extracts of Tulsi (a) and Neem (b).

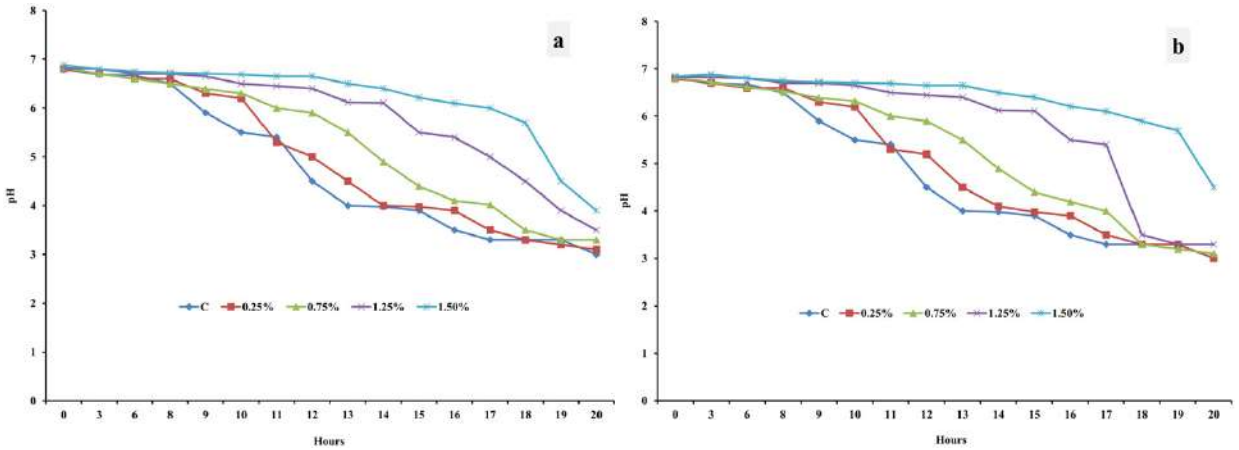

Fig. 2. Changing pattern of pH during preservation of raw milk treated with leaf aqueous extracts of Tulsi (a) and Neem (b).

Table 5. Clot-on-Boiling (COB) test in response to Tulsi and Neem aquous extract

Hours	Control	Tulsi (%)				Neem (%)			
	0%	0.25	0.75	1.25	1.5	0.25	0.75	1.25	1.5
0	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-
10	+	-	-	-	-	-	-	-	-
11	+	-	-	-	-	+	-	-	-
12	+	+	-	-	-	+	-	-	-
13	+	+	-	-	-	+	-	-	-
14	+	+	+	+	-	+	+	-	-
15	+	+	+	+	+	+	+	-	-
16	+	+	+	+	+	+	+	+	-
17	+	+	+	+	+	+	+	+	+

(-) = COB Negative, (+) = COB Positive.

Table 6. Total Viable Count (log_{10}CFU/mL) of the raw milk treated with Tulsi and Neem aqueous extract at different concentrations

Hours	Control	Tulsi				Neem				p-value
	0%	0.25%	0.75%,	1.25%	1.5%	0.25%	0.75%	1.25%	1.5%	
0	3.35±1.20	3.35±1.10	3.35±1.00	3.35±1.20	3.35±1.20	3.35±1.20	3.35±1.00	3.35±1.20	3.35±1.10	0.100
3	4.80^{a}±0.52	3.81^{bc}±0.60	3.52^{bc}±0.53	3.33^{c}±0.41	3.30^{c}±0.23	3.85^{bc}±0.23	3.59^{bc}±0.45	3.45^{bc}±0.56	3.39^{c}±0.38	0.010
6	5.11^{a}±0.23	4.12^{b}±0.22	3.58^{c}±0.32	3.34^{c}±0.22	3.37^{c}±0.22	4.10^{b}±0.23	3.90^{cd}±0.22	3.78^{cd}±0.23	3.70^{cd}±0.23	0.011
9	5.13^{a}±0.23	4.22^{b}±0.21	3.50^{c}±0.23	3.34^{d}±0.22	3.37^{d}±0.22	4.10^{b}±0.25	3.90^{c}±0.25	3.78^{c}±0.23	3.70^{c}±0.12	0.023
12	5.52^{a}±0.11	4.50^{b}±0.10	3.68^{c}±0.10	3.74^{c}±0.15	3.87^{c}±0.15	4.30^{b}±0.15	4.89^{b}±0.15	3.75^{c}±0.12	3.57^{c}±0.11	0.008
15	5.57^{a}±0.50	4.55^{b}±0.25	3.62^{d}±0.22	3.78^{d}±0.25	3.97^{cd}±0.25	4.39^{b}±0.25	4.92^{b}±0.25	3.89^{cd}±0.20	3.50^{b}±0.20	0.019
18	5.87^{a}±0.20	4.59^{ab}±0.20	3.82^{c}±0.20	3.79^{c}±0.20	4.12^{b}±0.33	4.59^{ab}±0.21	4.95^{ab}±0.20	4.52^{ab}±0.20	3.62^{ab}±0.20	0.012
20	5.11^{a}±0.02	4.59^{b}±0.07	3.82^{c}±0.07	3.79^{c}±0.17	4.12^{bc}±0.17	4.59^{b}±0.02	4.95^{b}±0.11	4.52^{b}±0.01	3.62^{b}±0.10	0.011

Mean ± SD.

Conclusion

In Bangladesh, milk losses its shelf-life very quickly due to high temperature and relative humidity. Herbal extracts can be used as an effective natural preservative for milk as it plays an important role in the LP system. The Tulsi and Neem leaves are available everywhere in Bangladesh. The majority percent of raw milk come from rural areas where the existing situations regarding raw milk preservation are difficult. This can be solved by using the herbal aqueous extract for prolonging the shelf-life of raw milk. Exploring the Tulsi and Neem leaf extracts in response to raw milk preservation, both extracts were found to have significant influence in extending the shelf- life. Thus, it can be recommended that both Tulsi and Neem with 1.5% concentration can be used to improve the shelf life of raw cow milk at room temperature.

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